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Biosafety assessment of transgenic poplars overexpressing xyloglucanase (AaXEG2) prior to field trials

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Abstract We performed biosafety assessments of transgenic poplars prior to field trials. Constitutive expression of the *Aspergillus aculeatus* xyloglucanase in *Populus alba* increased the cellulose content and specific gravity of its stem, the leaves of which were visibly greener, thicker, and smaller than those of the wild-type plant. Although the young transgenic poplars grew faster than the wild type in a growth chamber, there was no distinguishable difference in growth between the poplars when they were placed in a special screened greenhouse. Allelopathic tests showed that the transgenic poplars do not produce harmful substances. Based on all the biosafety assessments and the scientific literature on poplar species, we came to the conclusion that transgenic poplars probably do not disturb the biological diversity of the surrounding environment, even when they are submitted to field trials.

Key words Transgenic poplars · Xyloglucanase · Biosafety assessment · Greenhouse · Field trial

Introduction

Forest area on the earth has been reduced by two thirds in the past 10000 years, and is still decreasing mostly due to increasing logging operations. The decrease in forests is derived from human activities to use wood for paper, materials, and energy, etc. Reforestation has been required largely for sustainable industrial forests after deforestation not only to continue production of wood but also to protect natural forests. Reforestation also enhances CO_2 fixation on the earth and combats global warming because forests form a biological sink of CO_2. Genetic engineering could be used in forestry as a potential breeding technology to add useful traits such as an enhancement of both biomass production and improvement of wood properties because it is difficult to improve woody plants with long generations by conventional breeding. On the other hand, living modified organisms (LMOs) appear to have adverse effects on biological diversity, and may include harmful substances that interfere with wild plant growth and indirectly affect wild animal life by changing the base of the ecosystem. Therefore, the Cartagena Protocol was internationally adopted in 2000 to avoid the adverse effects of LMOs on biodiversity. In Japan, the protocol was ratified in 2003, and domestic laws and related regulations were enacted in 2004, controlling the use of LMOs.

When developing a new transgenic plant, the transformants are fast-grown under well-controlled and contained conditions in a climatic growth chamber or a glasshouse in order to assess whether the respective traits are expressed. Once transformants have been confirmed, they need to be assessed under near-field conditions to evaluate the influence of environmental factors before a field trial. In Japan, the biosafety assessments of transgenic plants have been defined and executed under regulatory guidelines in four stages: (1) either growth chamber or containment glasshouse trials under controlled conditions; (2) special screened greenhouse trials under semicontrolled or near-field conditions; (3) experimental field trials in a confined field with a security fence, which is called an "isolated field" in Japan; and (4) ordinary field trials. The present communication describes the biosafety assessments of transgenic poplar plants prior to experimental field trials.

The genus *Populus* contains between 22 and 75 species, depending on which taxonomic classification is considered,[1]

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although it is reasonable to assume approximately 30 species.[2] This genus is widely distributed throughout the northern hemisphere in both the temperate and subtropical zones,[3] and is suitable as a biomass resource due to its fast growth and short rotation cycle. *Populus* species are easily transformed compared with many other tree species. Because this genus has a small genome size, the genome project has been carried out for *Populus trichocarpa*.[4] Thus, *Populus* is a suitable model system for genetic engineering of trees with commercial values as wood materials.[2] We produced transgenic poplars (*Populus alba*) that could grow quickly with high cellulose content and elevated specific gravity by overexpressing a xyloglucanase (AaXEG2) from *Aspergillus aculeatus*.[5] The transgenic poplars are expected to be effective as a wood resource for pulp and paper materials because of their high cellulose content and density. Prior to the field trial of the transgenic poplars, we examined the biosafety assessments of the transgenic poplars on biological diversity in accordance with the domestic regulations (http://www.bch.biodic.go.jp/english/law.html).

Materials and methods

Plant material

Leaves of aseptically grown *Populus alba* plants, which were thought to be female, were cocultivated with *Agrobacterium tumefaciens* LBA4404 harboring binary vector pBE2113-AaXEG2. The vector consisted of the gene for *Aspergillus aculeatus* xyloglucanase *AaXEG2* (accession number AY160774) with *PopCel1* signal peptide[6] under control of the CaMV35S promoter and E12Ω enhancer sequences, and the gene for kanamycin resistance *nptII* under control of the nopaline synthase promoter.[5] After cocultivation, the transgenic poplar was regenerated on adventitious shoot regeneration medium containing selection antibiotics (kanamycin) and disinfection antibiotics (carbenicilline). The regenerated transgenic poplars and control wild-type poplars were propagated by cuttings and grown in a growth chamber, a containment glasshouse, and a special screened greenhouse, successively, and used as plant materials for examining the traits of transgenic poplars.

Molecular analysis of transgenic poplars

For polymerase chain reaction (PCR) analysis, genomic DNA was extracted from leaves of each line of transgenic and control wild-type poplar grown in the greenhouse. The oligonucleotides used for forward primers had the nucleotide sequence 5′-CGGTGACTTCACCCTGTACAACG-3′ (nucleotide position 137–160), which was homologous to the coding strand of the chimeric gene for the *PopCell* signal peptide and the *AaXEG2* protein; that for reverse primers, 5′-GGCCGCGAATTCACTAGTGATTCTCC-3′ (nucleotide position 815–840) was complementary to each coding strand. The PCR reaction was initially denatured at 94°C for 5 min and in the subsequent cycles at 94°C for 15 s. Annealing and elongation cycles were 30 s at 63°C, and 1 min at 68°C, respectively. After these cycles were repeated 30 times, PCR products were size-separated by electrophoresis in a 0.9% agarose gel. For genomic Southern hybridization, the purified genomic DNA extracted from the leaves was fully digested with *Hin*dIII, fractionated on a 0.8% agarose gel, hybridized with a PCR-DIG-labeled probe including the full coding sequence of *AaXEG2*, and detected by chemiluminescence. Western blot analysis was performed as described before.[5] The protein for the blotting was extracted from plants grown in the growth chamber and the greenhouse.

Measurement of cell wall component and determination of specific gravity of stems

The amount of cellulose in secondary xylem of the tenth internode was measured from the fraction that was insoluble in ethylenediaminetetraacetic acid (EDTA) and 24% KOH.[5] Methylation analysis for determination of xyloglucan content and measurement of specific gravity were performed as described by Park et al.[5]

Growth measurement and observation of leaf morphology

The height of four individual plants from each of the transgenic lines and wild type grown in a special screened greenhouse was measured for ca. 4 months. The diameter of the stem at a height of 10 cm from the ground was also measured. A fully expanded and typical leaf of each line grown in the greenhouse was photographed.

Allelopathic analysis of transgenic plants

We analyzed three allelopathic effects. First, the effect of secreted substances from poplar roots on the test plants was analyzed by the plant box method.[7] The poplar for examination was propagated by cuttings and grown for 3 months in a containment glasshouse. The poplar was then planted in 0.75% agar in a plant box in conjunction with test plant lettuce (Great Lakes 366), and the root growth of the lettuce was evaluated. Second, the effects of secreted substances from poplar roots on microorganisms (i.e., fungi, actinomycetes, and bacteria) in the soil were analyzed by the dilution plate method[8] based on plate counts. This is an evaluation of the soil microbial community in the soil used to grow transgenic or wild-type poplar for 5 months in a special screened greenhouse. Third, the effects of substances in dead poplar plants on the test plants were analyzed by the plow-in method[9] using leaf debris of transgenic and wild-type poplars grown for 5 months in a special screened greenhouse. Preweighed samples (180 g) of soil were mixed with 0, 1, 2, and 4 g of dried leaf debris of the poplar. Then, seeds of test plant lettuce were planted in the soil, and the root growth of the lettuce was evaluated.

Table 1. Xyloglucanase activity, xyloglucan content, cellulose content, and specific gravity of transgenic poplars (trg300-1, trg300-2) and wild type

Line	Xyloglucanase activity (unit/mg protein)	Xyloglucan content (μg/g dry weight)	Cellulose content (mg/g dry weight)	Specific gravity
trg300-1	234 a	5.3 (0.2) a	467 (4.8) a	0.36 (0.01) a
trg300-2	283 a	5.9 (0.3) a	458 (8.0) a	0.36 (0.01) a
Wild type	9 b	82.0 (2.5) b	420 (5.4) b	0.31 (0.02) b

Data are given as averages and standard deviation ($n = 5$). Values in same column with different letters are significantly different by least significant difference test ($P < 0.05$)

Glyphosate herbicide treatment

In order to confirm whether glyphosate herbicide [the active ingredient is 41% isopropylamine salt of *N*-(phosphonomethyl) glycine] kills poplar plants, we sprayed a fivefold or tenfold dilution of the herbicide on the leaves and stems of the wild-type poplar plants. The height of the plants was ca. 40 cm and the plants were grown in a glasshouse.

Results and Discussion

Transgenic poplars

Two lines (trg300-1 and trg300-2, Table 1) in which xyloglucanase activities in the cell-wall-bound fraction of the stem were more than 25-fold that in the stem of the wild type were selected from 54 independent transgenic poplar lines that expressed *Aspergillus aculeatus* xyloglucanase (AaXEG2) under the control of a constitutive promoter.[5] The two lines were subcultured by shoot-tip culture for approximately 1 year with a 4-week interval, and then propagated two times by stem cuttings. In order to check whether live *Agrobacterium tumefaciens* existed in the transgenic poplars, the transgenic poplar plants were homogenized and incubated in YEB medium at 28°C for 48 h. The bacterium *A. tumefaciens* was not found on the medium plated with the homogenates of the plant tissues (Fig. 1), indicating the fact that the bacterium was not alive and did not remain in the transgenic poplars.

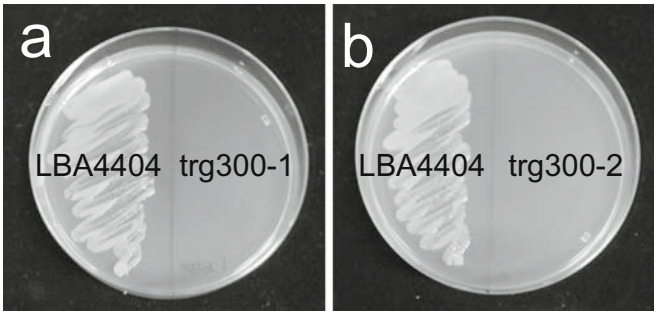

Fig. 1a, b. Examination of the survival of *Agrobacterium tumefaciens* LBA4404 in transgenic poplars. Homogenized transgenic poplar trg300-1 (**a**) and trg300-2 (**b**) were plated in YEB medium. After incubation the bacteria were not detected in the YEB medium plated with either transgenic poplars

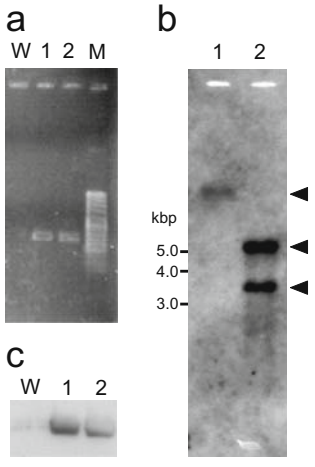

Fig. 2a–c. Molecular analysis of transgenic poplars. Transgene *AaXEG2* was detected by **a** polymerase chain reaction (PCR) analysis and **b** genomic Southern analysis in the genomes of transgenic poplars propagated by cuttings. Southern analysis showed that one copy and two copies of *AaXEG2* were integrated in the genomes of trg300-1 and trg300-2, respectively (*arrowheads* indicate hybridized signals). **c** Western blot confirmed that AaXEG2 was expressed stably in transgenic poplars propagated by cuttings. *W*, wild type; *1*, trg300-1; *2*, trg300-2; *M*, size marker

Existence of transgenes and stability of their expression

As shown in Fig. 2a, transgenic poplar lines displayed the amplification of the PCR fragments at the expected size of 700 bp. Genomic Southern hybridization showed that the transgene might exist as one copy for trg300-1 and two copies for trg300-2, respectively, at different loci (Fig. 2b). The genes, which are integrated in genomes at low copy number as in these transgenic poplars, are expected to be expressed stably. Indeed, Western blot analysis showed that *A. aculeatus* xyloglucanase was expressed in the leaves of both transgenic poplar lines grown in the greenhouse, running at a position corresponding to the size (28 kDa) of the mature xyloglucanase (Fig. 2c). These results indicate that *AaXEG2* was integrated into the poplar genomes and expressed stably in individuals propagated by cuttings.

Growth and leaf morphology of transgenic poplars

The stem height (Fig. 3a) and diameter (Fig. 3b) on each date measured, except for the diameter on July 31, were not significantly different between the wild type, trg300-1, and trg300-2, as determined by one-way analysis of variance (ANOVA) ($P > 0.05$). Therefore, the growth of the transgenic plants was thought to be similar to that of the wild-type plants in the special screened greenhouse, although all the elongations and diameter increments of the transgenic shoots were faster than those of the wild type for young plants in the climatic growth chamber.[5] The leaves in both lines of transgenic plants were visibly greener, thicker, and smaller than those of the wild type (Fig. 4), and resembled

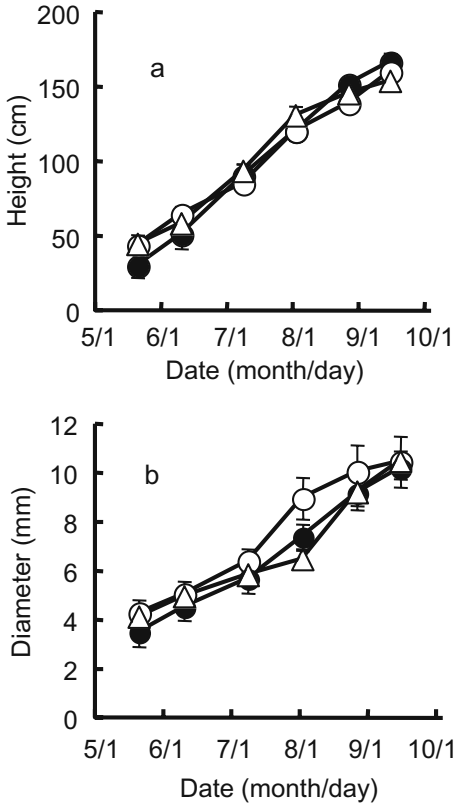

Fig. 3a, b. Growth of transgenic poplars (trg300-1 and trg300-2) and wild-type poplar in a special screened greenhouse for 4 months. **a** Height and **b** diameter growth represent the mean of four independent plants for each line. *Error bars* represent standard deviations. *Filled circles*, wild type; *open circles*, trg300-1; *open triangles*, trg300-2

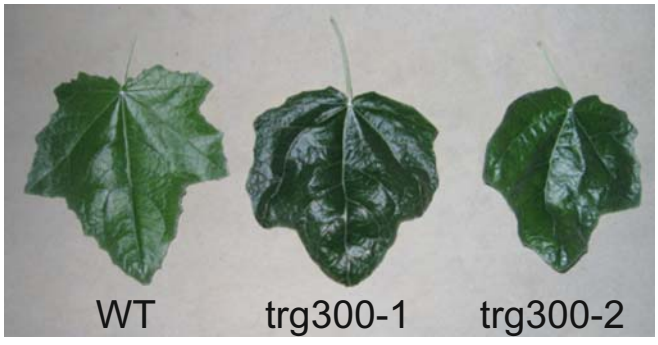

Fig. 4. Leaves of the wild-type (*WT*) and the transgenic poplars (trg300-1 and trg300-2)

sun leaves, which grew as if under strong sunlight. The leaf phenotype of the transgenic lines could be explained as a result of greater expansion of leaf palisade parenchyma cells and less air space.[5]

Cell walls

The amount of xyloglucan in the stems of the transgenic lines was only about 7% of that in the wild-type plant (Table 1), confirming that xyloglucanase (AaXEG2) expressed in the stems hydrolyzed xyloglucan in the walls of the transgenic poplars. Nevertheless, the cellulose content and specific gravity of the xylem of the transgenic lines were about 10% and 16%, respectively, higher than the wild type (Table 1). It seems likely that, because xyloglucan firmly crosslinks cellulose microfibrils, cellulose biosynthesis is restricted by xyloglucan crosslinkings. Therefore, the degradation of xyloglucan increased cellulose content in the xylem and its specific gravity by increasing the density of cellulose in the transgenic poplars.

Allelopathic analysis of transgenic plants

The secreted substances from roots and leaves were examined by measuring lettuce root elongation and evaluating the soil microbial community in terms of their allelopathic effects on other organisms. As shown in Fig. 5a, the elongation was slightly higher in trg300-1 and trg300-2 than that in the wild type but not significantly, as determined by one-way ANOVA ($P = 0.197$). This indicates that the effects of root secreta on growth of other plants did not differ between the transgenic and the wild-type poplars. One-way ANOVA of the data shown in Fig. 5b revealed no significant differences between the wild type, trg300-1, and trg300-2 in number of fungi, actinomycetes, or bacteria ($P = 0.07$, 0.58, and 0.29, respectively) by the diluted plate method, indicating that the effects of root secreta on microorganisms in the soil did not differ between the transgenic and the wild-type poplars. One-way ANOVA of the data shown in Fig. 5c revealed no significant differences in the root elongation of the test plant in the presence of leaf debris (1, 2, and 4 g dry weights with $P = 0.27$, 0.51, and 0.80, respectively) of wild type, trg300-1, and trg300-2, indicating no difference in the effects of leaf secreta on the test plant between the transgenic and the wild-type plants.

Glyphosate herbicide treatment

After glyphosate was sprayed on both the leaves and stems of the wild-type poplar plants, the whole plant bodies died. No shoots regenerated at all, even from the roots, once the stem was treated with the herbicide. These results indicate that herbicide sprayed on the leaves and stem of the transgenic poplar will kill not only the parts above ground but also the parts of the plant underground.

Assessment

According to domestic regulations in Japan, assessment must be carried out for each of the following four items: competitiveness, productivity of harmful substances, crossability, and other properties. Competitiveness is a property of competition and interference with wild plants for resources such as nutrients, sunshine, and habitat, etc. Productivity of harmful substances is a property of transgenic product interference with the livelihood and growth of wild

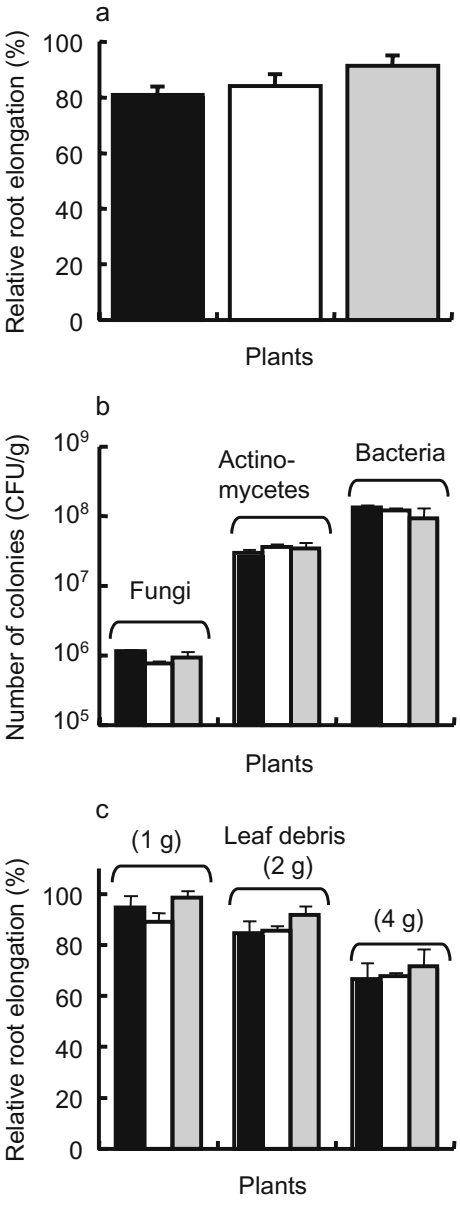

Fig. 5a–c. Allelopathic potential of the transgenic (trg300-1 and trg300-2) and the wild-type poplars. The effect of root secreta on the test plants (**a**), the effect of root secreta on microorganisms (fungi, actinomycetes, and bacteria) in soil (**b**), and the effect of leaf debris on the test plants (**c**) were analyzed by the plant box method,[6] dilution plate method,[8] and plow-in method,[9] respectively. *Error bars* represent standard deviations. *Filled bars*, wild type; *open bars*, trg300-1; *gray bars*, trg300-2

plants and animals or microorganisms. Cross-ability is a property of hybridization with wild plants and transmission of foreign genes. Others are properties, that is, indirect effects on wildlife by altering the base of the ecosystem, that are considered to adversely influence biological diversity.

Competitiveness

Populus species in the section *Leuce*, which includes *Populus alba*, the host plant of the transgenic poplars, naturally reproduce mainly by vegetative reproduction. They regenerate uniformly by root suckers in disturbed land such as burnt sites, but the number of individuals decreases with time due to the dominance of other tree species.[10] This indicates that *P. alba* is a typical pioneer tree species. By constitutive expression of the *A. aculeatus* xyloglucanase gene in poplars, cellulose content and specific gravity of the stem were significantly increased (Table 1), and posture control ability by growth stress in tension wood decreased compared with the wild type (Baba, unpublished data). However, these wood properties of transgenic poplars do not enhance the competitiveness of the host plant. The leaves of transgenic poplars were visibly greener, thicker, and smaller than those of the wild type (Fig. 4), with the potential to change their growth rate through variable photosynthetic ability. Nevertheless, there was no difference in growth rate between the transgenic and the wild-type plants in a special screened greenhouse (Fig. 3), indicating that the competitiveness of the transgenic poplars was not enhanced.

Productivity of harmful substances

Poplar species including *P. alba*, the host plant of the transgenic poplars, should not produce any substances harmful to biological diversity in Japan, although some allelopathic effects were reported.[7,11] Xyloglucanase AaXEG2 is not a harmful substance to humans because the enzymes from *A. aculeatus* are used for enzymatic processing in food industries (http://www.ffcr.or.jp/zaidan/MHWinfo.nsf/a11c0985ea3cb14b492567ec002041df/c3f4c591005986d949256fa900252700?OpenDocument). The recombinant enzyme catalyzes the hydrolysis of xyloglucan into its oligosaccharides specifically in the cell walls of poplars, and is not thought to affect other metabolisms in the plant bodies. Allelopathic analysis (Fig. 5) indicates that there is no significant difference in productivity of harmful substances among the transgenic and the wild-type poplars.

Cross-ability

The host poplar species of transgenic poplars is a member of section *Leuce*. Poplar species that are included in section *Leuce* and grow naturally in Japan are *Populus sieboldii* Miq. and *Populus tremula* L. var. *davidiana* Schneid,[12] both of which can cross with the host (*P. alba*). Hybrids among section *Leuce* are also able to cross with the host.[13] Thus, the foreign genes transferred to transgenic poplars could transmit to the wild poplar species by crossing if transgenic poplars are matured to flowering. However, because it takes 10–15 years for poplars (*P. alba)* to flower,[14] it is unlikely that the transgenic poplars would have flowers during the 5-year field trial. Therefore, we have assessed a low probability of cross pollination among the transgenic poplars and other wild poplar species when planted in the field for 4 years.

Other properties

Cellulose content and specific gravity of the transgenic poplars are significantly higher than those of the wild type (Table 1), and their leaves are visibly greener, smaller, and thicker than those of the wild type (Fig. 4). Poplars coexist with a wide range of insects (e.g., foliage insects of Lepidoptera and Coleoptera, and borer insects of Coleoptera) and are associated with many fungi.[3] The properties of the transgenic poplars in the confined experimental field should not affect biological diversity indirectly, even if the level of damage on the transgenic poplars caused by these insects and fungi may increase, compared with that of the wild-type poplars. The reason is that we can control these insects and fungi in the confined experimental field if the damage is so serious to be thought that the insects or fungi will affect the biological diversity of the surrounding environment. Furthermore, in order to assess the effect of the transgenic poplars on biological diversity in an ordinary field, we will compare the level of insect or fungus damage on the transgenic poplars and that of the wild-type poplars in the confined experimental field.

Conclusions

Depending on the assessment of the four items (competitiveness, productivity of harmful substances, cross-ability, and other properties) described above, we have concluded that the transgenic poplars overexpressing xyloglucanase (AaXEG2) should not affect the biological diversity, even when they are introduced into the confined experimental field. This assessment was also discussed at a Japanese governmental council organized by the Ministry of Agriculture, Forestry, and Fisheries and the Ministry of Environment, and the "Type 1 Use Regulations" for the field trial of transgenic poplars (http://www.bch.biodic.go.jp/english/lmo_2007.html) was approved.

We started the field trial for the transgenic poplars on March 26, 2007 and expect to complete the trial on December 31, 2011. Thus, the transgenic poplars will be examined for 4 years in an experimental field. It should be noted that the field is surrounded by a 8-m long fence with a 1-m deep underground steel-reinforced concrete wall, which is needed to prevent the roots from extending outside during the field trial, because the horizontal growth of the roots of mature trees can be remarkable.[15]

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